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## Original Article

# Simultaneous Immunoenzymatic Staining of Catecholamines, Catecholamine-biosynthesizing Enzymes, and Bromodeoxyuridine in Adrenal Medullary Cells of the Rat<sup>1</sup>

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The rat adrenal medulla consists mainly of low proliferating, highly differentiated parenchymal cells. By immunocytochemical techniques, two types of parenchymal cells can be identified, norepinephrine (NE)- and epinephrine (E)-storing cells. Bromodeoxyuridine (BrdU), a thymidine analogue often used to identify proliferating cells, can also be detected by immunocytochemical techniques. We developed double- and triple-labeling procedure(s) for simultaneous visualization of NE, E, dopamine  $\beta$ -hydroxylase (DBH), phenylethanolamine-N methyltransferase (PNMT), and BrdU in rat adrenal medulla. BrdU was administered to 7-week-old Wistar rats by mini-osmotic pumps. Tissues were fixed by perfusion with 4% paraformaldehyde and embedded in paraffin. By immunocytochemistry, first NE, E, DBH, and/or PNMT was detected by an indirect immunoperoxidase technique with Fast Red or Fast Blue as sub-

strate. Next, incorporation of BrdU was detected with an indirect immunoperoxidase procedure using diaminobenzidine (DAB). Both NE- and E-storing cells, as well as endothelial cells, can incorporate BrdU, i.e., are able to divide. Occasionally, we also found BrdU-stained mitotic figures in E, PNMT and DBH immunoreactive cells. No BrdU incorporation was found in the post-ganglionic neurons of the adrenal medulla. The procedures described enable a detailed cell kinetic study of the NE- and E-storing cells in the adrenal medulla, particularly in the rat, which can lead to a better understanding of cell renewal in the adrenal medullary tissue under normal and pathological conditions. (*J Histochem Cytochem* 43:39-46, 1995)

KEY WORDS: Adrenal medulla; 5-bromo-2-deoxyuridine; Norepinephrine; Epinephrine; Simultaneous immunostaining; Cell proliferation; Rat.

## Introduction

Cell kinetic studies based on mitotic counts have suggested that the parenchymal cells of the adrenal medulla of adult rats are not able to divide (Mitchell, 1948; Jackson, 1919), i.e., are terminally differentiated. Malvaldi et al. (1968), however, showed that in the chromaffin cells of the adrenal medulla mitotic figures occur if mitotic arrest is induced with colchicine. Messier and Leblond (1960) also found low proliferative activity in adrenal medullary cells using [<sup>3</sup>H]-thymidine incorporation during the S-phase of the cell cycle.

Bromodeoxyuridine (BrdU), a thymidine analogue incorporated during DNA replication, can easily be detected by immunocytochemical techniques (Gratzner, 1982). This principle has been adopted to identify cells that are exposed to BrdU during the S-phase or are derived from previously BrdU-labeled cells. Since its introduction, this method has been used for cell kinetic studies in several

types of tissue (e.g., Patel et al., 1993; De Bruïne et al., 1992; Hyatt and Beebe, 1992; Soriano and Del Rio, 1991; Frederiks et al., 1990; Magaud et al., 1989; De Fazio et al., 1987; Schutte et al., 1987a; Dolbeare et al., 1983). Especially in slowly proliferating tissues such as the adrenal medulla, this technique has opened new possibilities to detect cell proliferation. Thus, for example, by continuous BrdU administration to adult rats for 73 days using mini-osmotic pumps in the adrenal medulla, about 40-50% of parenchymal cells appeared to be labeled (Verhofstad, 1993). These labeled cell nuclei were randomly localized throughout the adrenal medulla just like the mitotic figures described by Malvaldi.

The parenchyma of the adrenal medulla of most mammals consists of two different cell types synthesizing and storing either norepinephrine (NE) or epinephrine (E) (Coupland, 1971, 1989). Among other techniques, these cells can be identified by immunocytochemistry using antibodies to NE and E (Verhofstad et al., 1980, 1983) or the biosynthesizing enzymes dopamine  $\beta$ -hydroxylase (DBH) and phenylethanolamine-N methyltransferase (PNMT) (Hököfelt et al., 1973; Goldstein et al., 1971) (see also Table 3). With both sets of antibodies, i.e., antibodies to NE and E or DBH and PNMT, identical results can be obtained. However, antibodies to

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DBH and PNMT do not always crossreact with the enzymes of other species to the same extent, or may even not crossreact at all. Therefore, antibodies to NE and E, molecules that are not immunogenic by themselves, provide a more universal procedure to identify NE- and E-storing cells (for detailed discussion see Verhofstad et al., 1983, 1985, 1989). Limited efforts to combine BrdU cytochemistry and methods to identify NE- and E-storing cells have been reported. Only Tischler described the combined staining of BrdU and PNMT (Tischler et al., 1989) or tyrosine hydroxylase (Tischler et al., 1992) in rat adrenal medullary cells.

In the present study we describe immunocytochemical staining procedures that allow simultaneous visualization of BrdU and one or two catecholamines or catecholamine-synthesizing enzymes. These double- and triple-staining procedures enable the demonstration of BrdU incorporation into the NE- and E-storing cells, offering new possibilities to study renewal of these cells in the adrenal medulla under normal and pathological conditions.

## Materials and Methods

**Animals.** Male Wistar rats (Charles River Wiga; Sulzfeld, Germany) weighing approximately 200 g and aged 7 weeks were used. During the BrdU labeling experiments they were housed in Macrolon Type 3 cages with water and standard laboratory food (RMH Hope Farms; Woerden, The Netherlands) ad libitum, maintained on a 12-hr light:dark cycle in air-conditioned rooms (20–23°C), and treated according to the institution's guidelines for care and use of laboratory animals.

**BrdU Administration.** BrdU (Serva; Heidelberg, Germany) dissolved in PBS (0.01 M, pH 7.4, with or without 0.37%  $\text{NH}_3$  to improve the solubility) at a concentration of 30 mg/ml was sterilized by a pyrogen-free microfilter (Millipore, 0.2  $\mu\text{m}$ ). The BrdU solution was given continuously to obtain a reasonable number of labeled cell nuclei in the adrenal medulla using Alzet mini-osmotic pumps (Alza; Palo Alto, CA) Type 2001 with a capacity of 200  $\mu\text{l}$  and a mean pumping rate of 1  $\mu\text{l/hr}$ . The filled pumps were weighed to check the amount of solution loaded. Before implantation the pumps were kept in a sterile isotonic saline solution at 37°C for at least 4 hr to obtain a steady-state pumping rate. Under semi-sterile circumstances and light ether anesthesia, the pumps were implanted IP in four rats and removed after 1 week. In two of these rats pumps were replaced to complete the 2-week administration of BrdU. At the end of each labeling experiment the peritoneal cavity of the animals was checked for the presence of inflammation and formation of adhesions.

**Tissue Preparation.** Rats without BrdU incorporation and rats at the end of a 1- or 2-week BrdU labeling period were anesthetized by IP injection of sodium pentobarbital (Narcovet; Arnhem, The Netherlands) (60 mg/kg bw) and fixed by perfusion through the left ventricle of the heart with ice-cold 4% paraformaldehyde (Merck 4005; Darmstadt, Germany) in 0.1 M phosphate buffer, pH 7.3 (Pease, 1962). After 15 min the adrenal glands and several other tissues were dissected and post-fixed in the same fixative for 2 hr at 4°C. The adrenals were then cut in two equal parts with stainless steel razor blades. Tissues were rinsed in PBS, dehydrated in a graded series of ethanol, rinsed in xylene two times for 30 min each and impregnated twice for 30 min with paraffin (Paraplast Plus) (Oxford Labware; St Louis, MO) under vacuum. Paraffin sections were cut at 4  $\mu\text{m}$  thick, mounted on glass slides with Tissue Adhesive (Laboratory-Service Benelux; Apeldoorn, The Netherlands), and kept overnight at 37°C to improve adhesion of the sections to the slides.

**Antisera.** A mouse monoclonal antibody (MAb) was used to stain BrdU. This MAb (clone II B 5) was prepared by Schutte et al. (1987a). To stain NE and E cells, polyclonal antisera to NE, E, DBH, and PNMT were used.

The preparation and characterization of these sera have been reported elsewhere (Verhofstad et al., 1980, 1983; Goldstein et al., 1971). All other antibodies were obtained from Dakopatts (Copenhagen, Denmark). Antisera were diluted in a 0.15 M PBS solution containing 2% bovine albumin (Sigma; St Louis, MO) and 0.1% Triton X-100 (Sigma) as indicated in Table 1.

**Staining Experiments.** Tissue sections were cleared from paraffin with two changes of xylene and rehydrated in a graded series of ethanol and distilled water.

BrdU detection was based on procedures described by Schutte et al. (1987a,b). After washing three times for 5 min in PBS the sections were pre-treated with pepsin (Boehringer Mannheim, Mannheim, Germany; lot no. 108057, 2500 U/mg) in 0.1 N HCl for 30 min at 37°C, rinsed in PBS (three times for 5 min), and then incubated at 37°C in 2 N HCl to obtain single-stranded DNA for the presentation of the antigenic BrdU sites. Subsequently, the pH was neutralized by washing twice in 0.1 M sodium tetraborate buffer (Merck), pH 8.5, and three times in PBS. Next, the sections were incubated overnight at 4°C with the mouse MAb to BrdU (1:50,000), rinsed in PBS (three times for 10 min), and incubated with peroxidase-conjugated rabbit anti-mouse IgG immunoglobulins (Dakopatts; 1:200) for 1 hr at room temperature (RT). The slides were then washed three times in PBS and the antibody binding sites visualized by submersing the slides in a peroxidase substrate. Finally the sections were counterstained with Mayer's hematoxylin. As a control, the MAb to BrdU was substituted by a mouse MAb that was not related to BrdU.

To detect NE, E, DBH, and PNMT, indirect immunoenzymatic techniques with peroxidase- and alkaline phosphatase-conjugated immunoglobulins were used. After three 10-min rinses in PBS (pH 7.4), slides were incubated with primary and conjugated antisera, if necessary preceded by one of the linking immunoglobulins, as indicated in Table 1. The sections were rinsed at RT with 0.1 M PBS, pH 7.4, before and after each incubation step (three times for 10 min). Next, they were exposed to a peroxidase (1–2 min) or alkaline phosphatase substrate (10–15 min). After rinsing in running tapwater (10 min), the peroxidase-stained slides were incubated for 1 min in a cupric sulfate ( $\text{CuSO}_4$ ) solution (0.5% NaCl in 0.5%  $\text{CuSO}_4$ ). Sections were counterstained with Mayer's hematoxylin.

Table 1. *Antibodies used for immunoenzymatic detection of NE, E, DBH, and PNMT<sup>a</sup>*

Primary antibodies	
Goat anti-bovine DBH 1:1600 (Goldstein et al., 1971)	
Goat anti-bovine PNMT 1:1600 (Goldstein et al., 1971)	
Sheep anti-norepinephrine 1:6400 (Verhofstad et al., 1983)	
Rabbit anti-epinephrine 1:3200 (Verhofstad et al., 1983)	
18 hr (overnight), 4°C	
Linking antibodies	
Fluorescein-conjugated rabbit anti-goat immunoglobulins (Dakopatts; code F 250, lot 074) 1:50	
Rabbit anti-sheep immunoglobulins (Dakopatts; code Z 181, lot 111) 1:100	
45 min, room temperature	
Conjugated antibodies	
Peroxidase-conjugated swine anti-rabbit immunoglobulins (Dakopatts; code P 217, lot 079) 1:40, 45 min, room temperature	
Alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (Dakopatts; code D 306, lot 121) 1:20, 30 min, room temperature	

<sup>a</sup> For details of the staining protocol for catecholamines and biosynthesizing enzymes, see Materials and Methods.



**Simultaneous Staining of NE and E.** Sections were incubated with a mixture of rabbit antibodies to E and sheep antibodies to NE, and rinsed in PBS. To stain E, sections were incubated with alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins, rinsed in PBS, and stained with Fast Blue substrate. Next, to stain NE, sections were rinsed in PBS, incubated with 10% normal rabbit serum, and rinsed in PBS. Finally they were incubated with alkaline phosphatase- or peroxidase-conjugated rabbit anti-sheep immunoglobulins (Dakopatts; code P163, lot 039), rinsed in PBS, stained with the Fast Red or AEC substrate, respectively, and rinsed in running tapwater.

**Simultaneous Staining of Catecholamines or Catecholamine-biosynthesizing Enzymes and BrdU.** For simultaneous immunocytochemical detection of BrdU and NE, E, DBH, or PNMT, we considered three possibilities: (a) staining of BrdU followed by staining of the cytoplasmic antigens; (b) staining of NE, E, DBH, or PNMT followed by detection of BrdU; and (c) detection of both antigens simultaneously using a mixture of the primary antibodies.

To determine which of the options was the most appropriate, a series of experiments were performed using the methods described above. First, by changing the concentration of pepsin in 0.1 N HCl from 0 (control) to 0.2 mg/ml and varying the incubation with 2 N HCl from 0 (control) to 4, 8, 15, 20, and 30 min, the optimal DNA denaturation procedure to stain BrdU was determined for different types of tissue. Second, the effects of these steps on the immunocytochemical staining of NE, E, DBH, and PNMT were analyzed. These experiments (see Results) showed that only option b, i.e., staining of NE, E, DBH, or PNMT followed by detection of BrdU, was feasible.

In detail, for the simultaneous detection of BrdU and NE, E, DBH, or PNMT in rat adrenal medulla, sections were first incubated with antibodies to NE, E, DBH, or PNMT, followed, in the case of NE, DBH, and PNMT, by one of the linking antibodies mentioned in Table 1. Sections were then incubated with alkaline phosphatase-conjugated immunoglobulins, rinsed in PBS, and stained with an alkaline phosphatase enzyme substrate and rinsed in running tapwater. To detect BrdU, sections were first rinsed in PBS and incubated in 0.05 mg/ml pepsin in 0.1 N HCl for 30 min at 37°C, then incubated in 2 N HCl of 37°C for 18 min and rinsed in 0.1 M sodium tetraborate buffer, pH 8.5, twice for 5 min. Next, sections were rinsed in PBS, incubated with BrdU antibody diluted in 0.15 M PBS containing 2% normal rabbit serum, 2% bovine albumin, and 0.1% Triton X-100 (Sigma), rinsed in PBS again, and incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins. Sections were then stained with the DAB substrate, rinsed in running tapwater, incubated in CuSO<sub>4</sub> solution, and rinsed again in running tapwater. If needed, sections were counterstained with Mayer's hematoxylin.

For simultaneous staining of BrdU as well as NE and E (triple labeling), sections were first processed according to the simultaneous staining procedure for NE and E, as described above, then rinsed in PBS and processed for detection of BrdU incorporation as described above.

**Enzyme Substrates.** The substrate solution used for staining of the peroxidase-labeled immunoglobulins was prepared by dissolving 0.65% diaminobenzidine tetrahydrochloride (DAB) (Sigma; D 5637) in 0.15 M PBS with 6.5% imidazole, to which H<sub>2</sub>O<sub>2</sub> (0.01%) was added just before use. Alternatively, 40 mg 3-aminoethyl-9-carbazole (AEC) (Aldrich Chemie; Brussels, Belgium) was dissolved in 10 ml *N,N*-dimethylformamide and added to 190 ml sodium acetate buffer, pH 4.8. Finally, 70 µl H<sub>2</sub>O<sub>2</sub> was added just before use.

For staining of the alkaline phosphatase-labeled immunoglobulins, the following solution was prepared: 4 mg naphthol AS-MX phosphate (Sigma; N-5000) in 20 ml of 0.2 M Tris with 0.02 mM MgCl<sub>2</sub>, pH 8.5, and 4.8 mg levamisole (Sigma; L-9756) to block endogenous alkaline phosphatase. Just before staining, 20 mg Fast Red TR salt (Sigma; F-1500) was added, and subsequently the solution was filtered and applied directly to the tis-

sue sections. A Fast Blue substrate was prepared by dissolving 2 mg naphthol AS-TR phosphate (Sigma; N-6125), 6 mg Fast Blue B (Sigma; D-3502) and 1.2 mg levamisole in 5 ml of 0.2 M Tris, 0.02 mM MgCl<sub>2</sub>, pH 8.5.

Stained sections were mounted in Kaiser's glycerol gelatin (Merck) and examined with a Zeiss Axiophot microscope. Black-and-white photomicrographs were made using Agfapan APX 25 professional film and printed on Kodak Polycontrast III RC photopaper. Color prints were taken from color slides made using Kodak Ektachrome 64 T film.

## Results

In the peritoneal cavity of the rats, no inflammation or formation of adhesions was observed after BrdU administration for 1 or 2 weeks.

### *BrdU Detection in Perfusion-fixed Tissue*

Sections were carefully screened to determine the precise localization and intensity of the staining reactions as well as the degree of structural preservation of cells and other tissue components. As shown in Table 2, we found that for detection of the incorporated BrdU, tissues fixed by perfusion with 4% paraformaldehyde revealed different optimal pepsin pre-treatment and DNA denaturation conditions. The adrenal medulla appeared to be much more sensitive to pepsin treatment than kidney and duodenum. At a pepsin concentration of 0.05 mg/ml the morphology of the adrenal medullary cells was already damaged, whereas in the kidney and duodenum cells were not affected at all. Differences also exist with regard to DNA denaturation with 2 N HCl. In the adrenal medulla BrdU staining remained intense only within a small time interval, i.e., 15–20 min. In the duodenum and kidney, however, 2 N HCl incubation time was less critical.

In all rats treated with BrdU, labeling was found throughout the epithelium of the duodenum, indicating that the BrdU treatment of the animals was adequate.

### *Effect of Pepsin and 2 N HCl on Immunocytochemistry of the Cytoplasmic Antigens*

By immunocytochemical staining of NE, E, DBH, and PNMT, NE- and E- storing cells could be identified, as indicated in Table 3. However, if the immunocytochemical labeling of NE and E, either by immunofluorescence or immunoenzymatic staining, was performed after pepsin and HCl pre-treatment in these cells, the nuclei instead of the cytoplasm became stained. Pepsin was found to be responsible for this phenomenon. At a concentration of 0.2 mg/ml, pepsin destroyed the cytoplasm completely, whereas the cell nuclei became intensely stained (Figure 2). At lower concentrations of pepsin the cytoplasm was less affected but the cell nuclei were also less

Table 2. Optimal pepsin and HCl pre-treatment to stain BrdU in cell nuclei of formaldehyde-fixed tissues<sup>a</sup>

	Adrenal medulla	Kidney	Liver	Pancreas	Duodenum
Pepsin (mg/ml)	0.02	0.05	0.05	0.1	0.1
2 N HCl (min)	15	8	30	30	30

<sup>a</sup> For details of the BrdU staining protocol, see Materials and Methods.



Table 3. Staining pattern of NE- and E-storing cells in adrenal medulla of the rat using immunocytochemical techniques<sup>a,b</sup>

	Immunoreactivity to			
	NE	E	DBH	PNMT
NE cells	++	-	++	-
E cells	+	++	++	++

<sup>a</sup> ++, strong reaction; +, moderate reaction; -, no reaction.

<sup>b</sup> DBH is the biosynthesizing enzyme that converts dopamine into NE and is therefore present in both cell types. PNMT is involved in the conversion of NE into E and is therefore present only in E cells.

intensely stained. Even with the pepsin concentration needed for adequate BrdU staining (0.02 mg/ml for adrenal medullary cells), cell nuclei still showed some staining for NE or E.

If the cytoplasmic antigens were first labeled by immunoenzymatic staining with DAB, AEC, Fast Red, or Fast Blue, treatment with pepsin or 2 N HCl did not affect or affected only slightly the staining properties of the NE and E cells. Only the DAB and AEC enzyme products were slightly bleached by 2 N HCl, whereas pepsin treatment showed no loss of staining quality.

### Double Labeling

The combination of Fast Red alkaline phosphatase staining of the cytoplasmic antigens NE, E, DBH, and PNMT, and DAB peroxidase staining of BrdU, gave the best contrast and staining quality. Both labeling procedures did not interfere except for the fact that the pepsin concentration and the duration of HCl treatment had to be adjusted slightly (see Materials and Methods). BrdU-positive cell nuclei could easily be recognized in alkaline phosphatase-labeled as well as unlabeled cells.

Figure 1 shows serial sections of a rat adrenal gland after 2 weeks of BrdU administration stained for BrdU and NE, E, DBH, or PNMT. Two types of adrenal medullary cells can be observed, i.e., NE-storing cells (only immunoreactive to NE and DBH) and E-storing cells (immunoreactive to NE, DBH, E, and PNMT) (see Table 3). Cell nuclei with BrdU incorporation are randomly localized throughout the adrenal medulla (Figures 1a and 1b). As shown in Figures 1c, 1d, and 1f, many E cells have incorporated BrdU and a few NE cells possess stained nuclei. In the adrenal medullary cells mitotic figures are rarely seen. In one of the double-labeling experiments a cell immunoreactive to E contained a mitotic figure stained for BrdU (Figure 3c). In serial sections the cytoplasm of this particular cell also stained positive for PNMT and DBH (not shown). Mitotic figures in NE-storing cells were not found.

For simultaneous labeling of NE and E, the combination of immunoenzymatic staining using AEC or Fast Red with Fast Blue as substrate gave good results. Figure 3a shows an alkaline phosphatase double labeling of NE and E in which first E is stained with Fast Blue followed by Fast Red staining of NE.

### Triple Labeling

By comparing simultaneous immunoenzymatic staining of NE, E, and BrdU and single-staining procedures, we found no interference between the three different labels. Figure 3b shows a section

of the adrenal medulla of a rat that had received BrdU for 2 weeks, stained according to the triple labeling procedure. Separate NE and E cells are clearly demonstrated. Some of the E cells had apparently incorporated BrdU.

### BrdU Staining Pattern of the Adrenal Medulla

Analysis of the sections stained according to the double- or triple-staining procedures described showed that, in addition to the NE- and E-storing cells, most of the endothelial cells also had incorporated BrdU. Ganglion cells were negative.

### Discussion

It is now generally accepted that in the adrenal medulla of adult rats two different cell types are involved in the biosynthesis of NE and E. These cells, the NE- and E-storing cells, can be identified by immunocytochemical techniques using antibodies to NE, E, DBH, and PNMT. As discussed elsewhere, application of antibodies to NE and E is not limited by the variable degree of crossreactivity encountered with the antisera to the biosynthesizing enzymes (Verhofstad et al., 1980,1983,1985,1989; see also Verhofstad, 1993).

Quantitative information on cell proliferation and cell loss is needed to understand why organs grow, decline, or stay in a steady-state situation. In addition, such information might be helpful to understand cell behavior during neoplastic growth and other pathological processes.

Procedures to demonstrate cell proliferation based on the immunocytochemical detection of BrdU incorporated into DNA during the S-phase of the cell cycle are well established (e.g., De Bruïne et al., 1992; Hyatt and Beebe, 1992; Frederiks et al., 1990; De Fazio et al., 1987; Schutte et al., 1987a). These procedures are also suitable to study cell proliferation in the rat adrenal medulla (Verhofstad, 1993).

To examine separately the proliferation rate of the NE- and E-storing cells in the adrenal medulla, we tried to combine the immunocytochemical techniques to identify NE- and E-storing cells and BrdU incorporation. If applied to two adjacent sections, in most cases it was hardly possible to determine the cytoplasmic characteristics of cells containing BrdU-stained nuclei. Therefore, we decided to perform both procedures on the same section. For practical reasons we decided to use paraffin-embedded material only.

For simultaneous staining of BrdU and cytoplasmic markers, previous authors have adopted double immunofluorescence (e.g., Penit, 1988; Watanabe and Raff, 1988; Houck and Loken, 1985), double immunoenzymatic procedures (e.g., Montuenga et al., 1992; Soriano and Del Rio, 1991; Harms et al., 1987; Schutte et al., 1987a), or a combination of immunofluorescence and immunoenzymatic staining (Magaud et al., 1989). In studying these options we noted that the procedures needed to obtain positive staining of BrdU in formaldehyde-fixed tissue reduced the antigenicity of NE, E, DBH, and PNMT. In addition, if these procedures were applied to sections previously stained with an indirect immunofluorescence technique, loss of fluorescence was observed. Therefore, we decided to explore only immunoenzymatic methods in detail.

In combining immunoenzymatic staining of NE, E, DBH, or PNMT and BrdU, several problems were encountered. First, because the immunogens used to raise antibodies to NE and E were



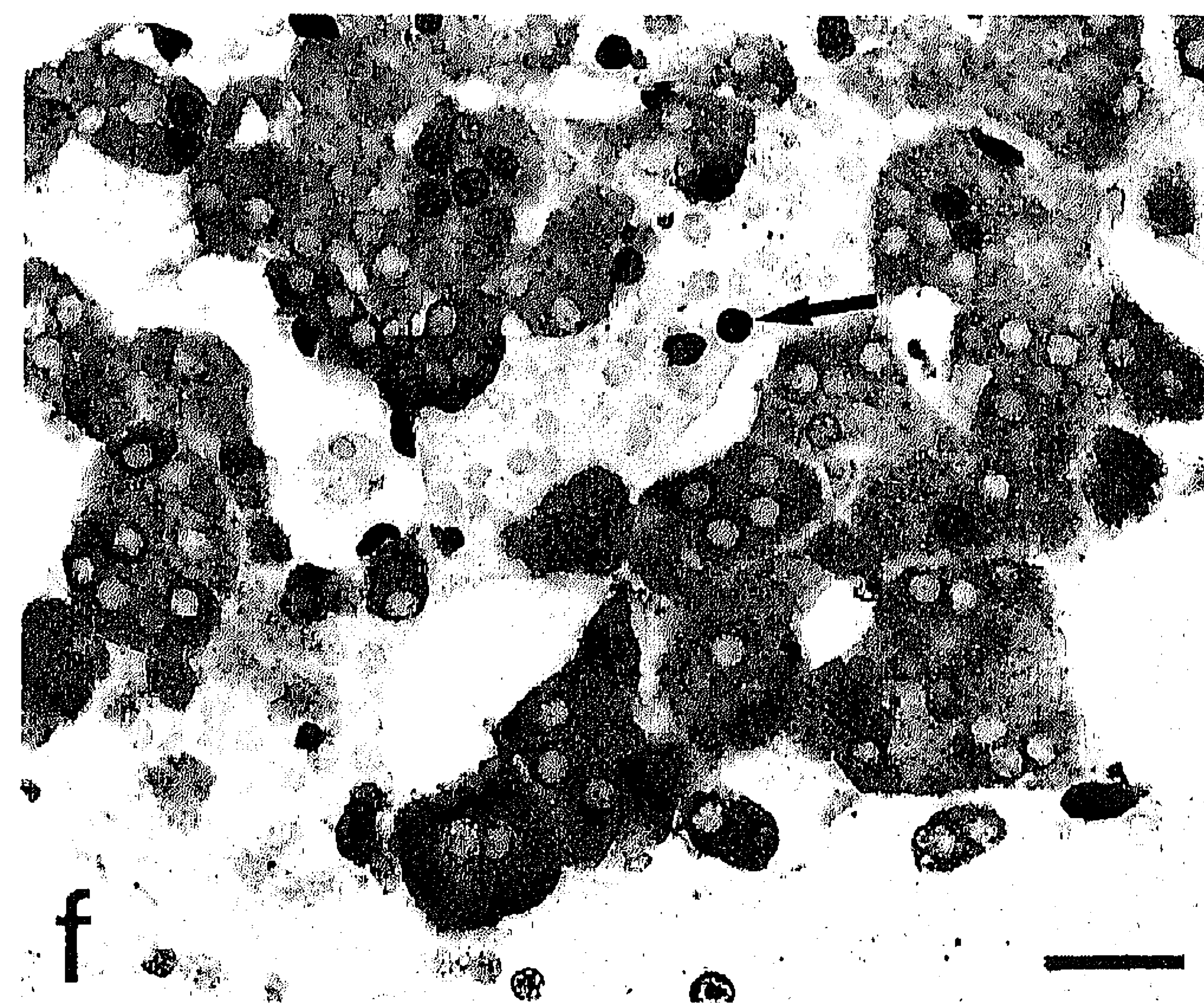
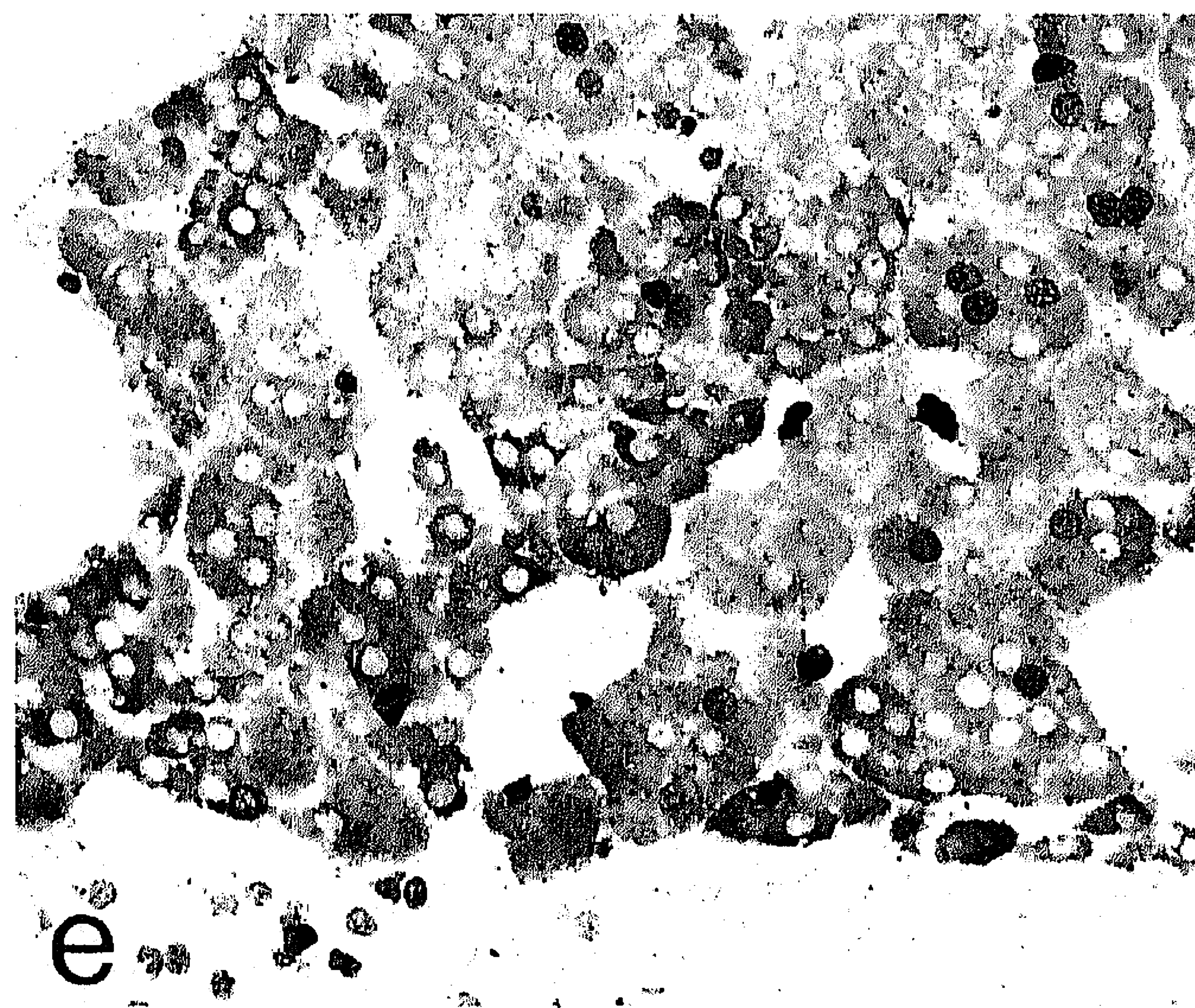
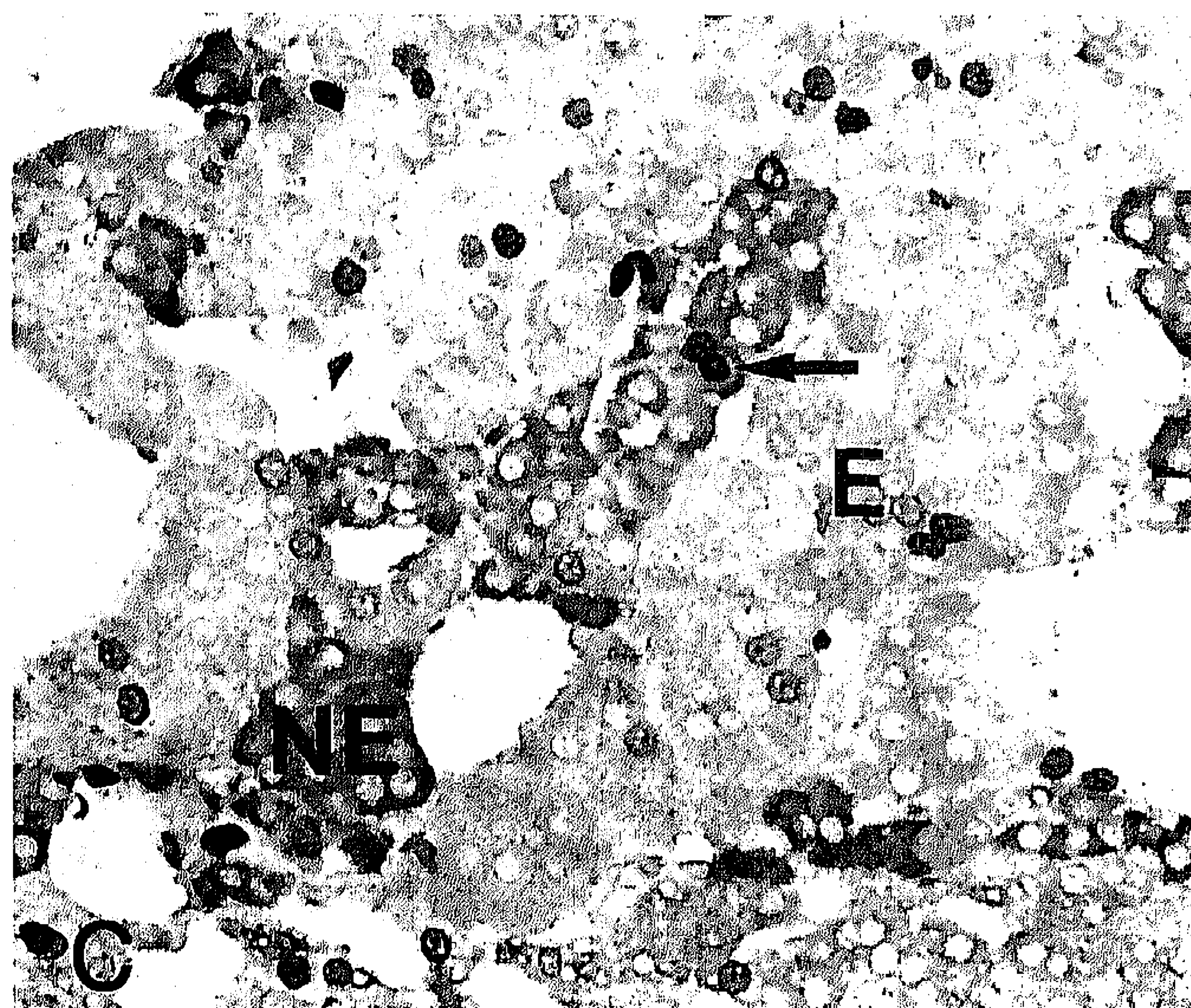
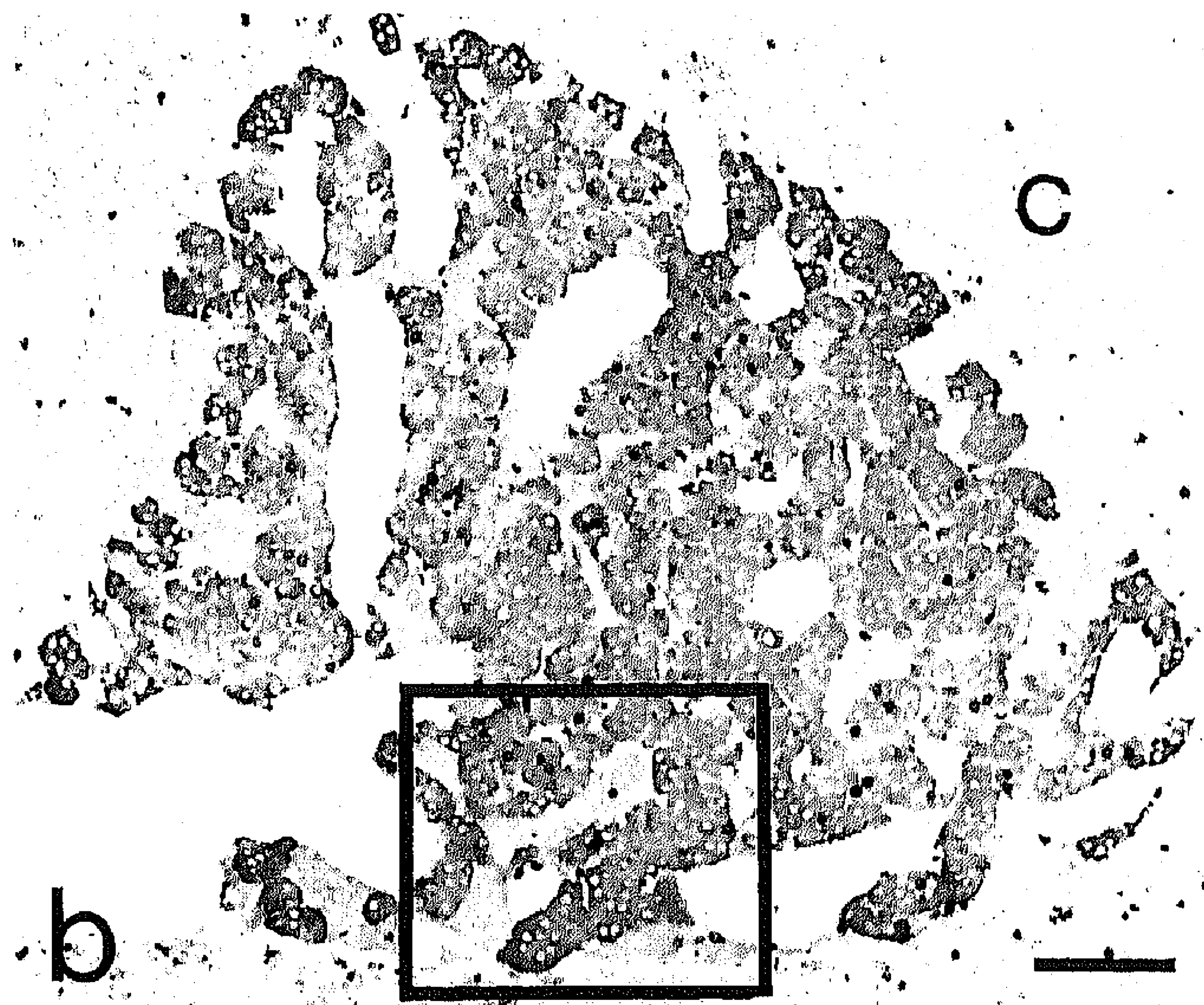
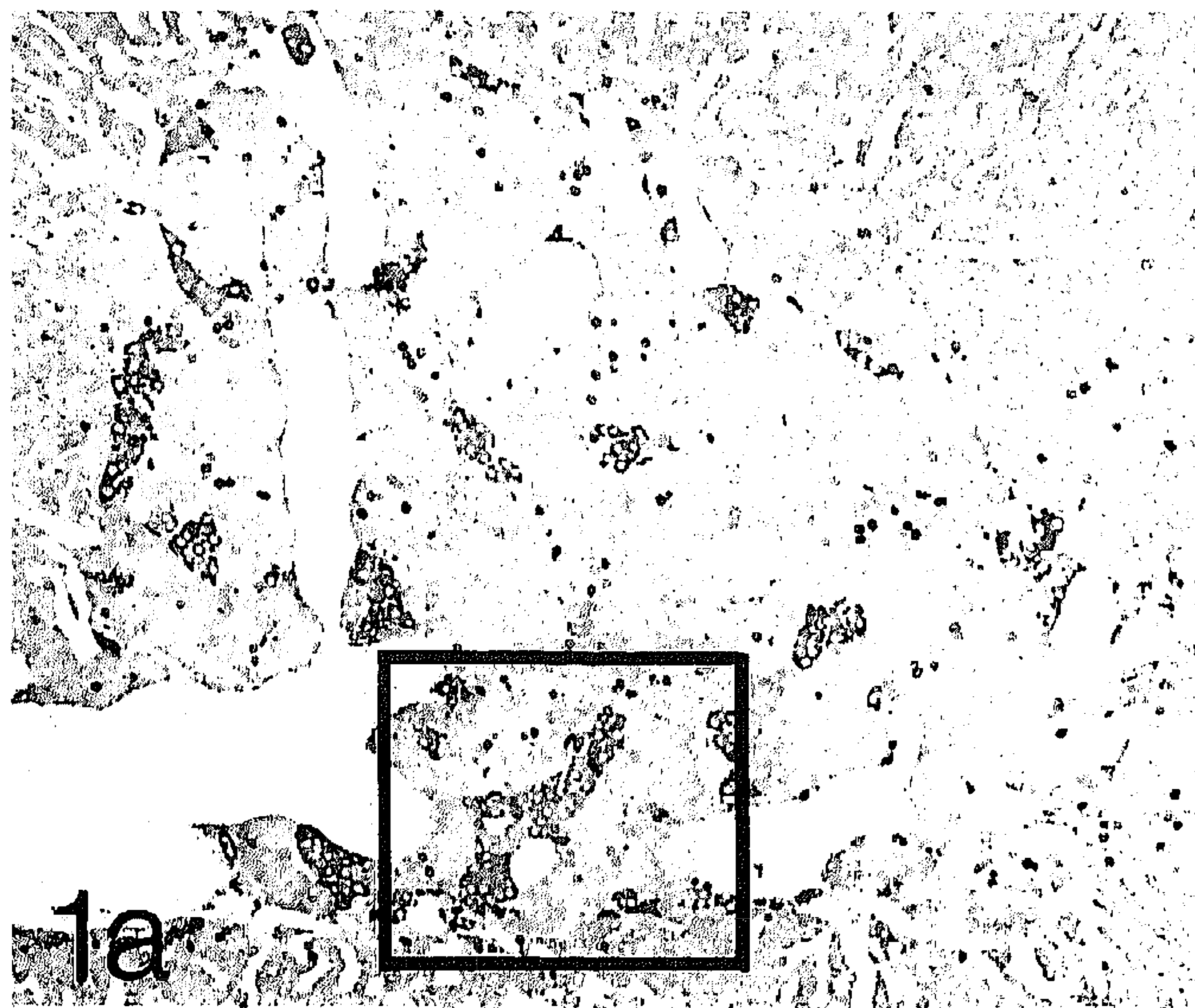


Figure 1. Rat adrenal gland exposed to BrdU for 2 weeks, fixed by perfusion with 4% paraformaldehyde, and embedded in paraffin. Serial sections (4  $\mu$ m) stained for bromodeoxyuridine and (a) norepinephrine, (b) epinephrine, (e) dopamine  $\beta$ -hydroxylase, or (f) phenylethanolamine-N methyltransferase. c and d are enlargements of areas indicated by rectangles in a and b, respectively. e and f are areas comparable to c and d. Long arrows (c,d,f) point to nuclei of norepinephrine cells (NE) with BrdU incorporation. Short arrow marks nucleus of endothelial cell with BrdU. C, adrenal cortex; E, epinephrine. Bars: a,b = 100  $\mu$ m; c-f = 30  $\mu$ m.



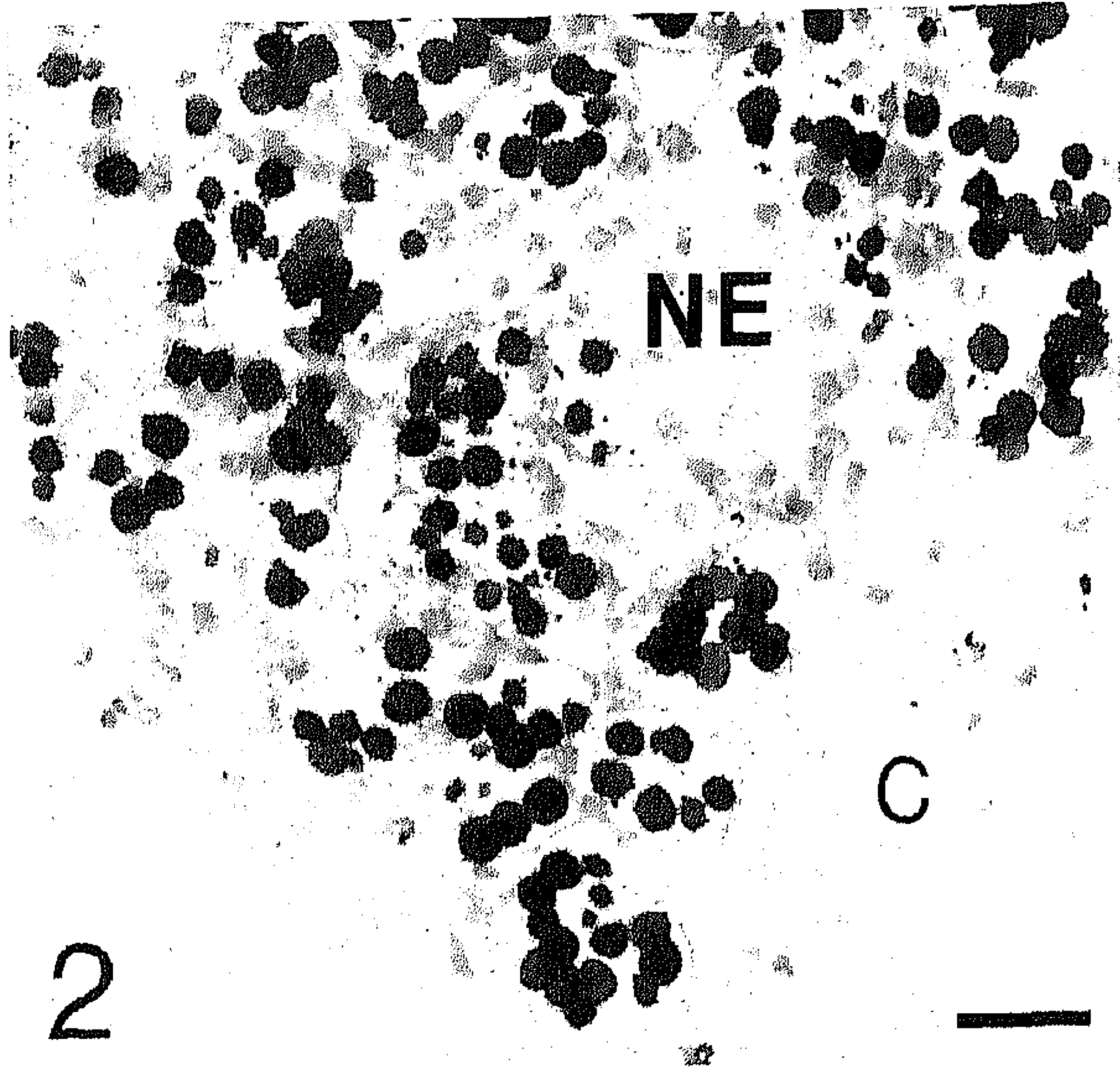


Figure 2. Rat adrenal gland exposed to BrdU for 2 weeks, fixed by perfusion with 4% paraformaldehyde, and embedded in paraffin. Section pre-treated with 0.2 mg/ml pepsin in 0.1 N HCl for 30 min at 37°C. Indirect immunofluorescence (FITC) staining with a rabbit antiserum to epinephrine. Note that nuclei instead of the cytoplasm of epinephrine-storing cells are stained. NE, norepinephrine-storing cells; C, adrenal cortex. Bar = 30  $\mu$ m.

synthesized using formaldehyde as a coupling reagent, to identify the NE- and E-storing cells tissue fixation with formaldehyde was needed (Verhofstad et al., 1980,1983). Previous studies have shown that under these conditions DBH and PNMT can be demonstrated as well (Verhofstad et al., 1985,1989). To detect BrdU other fixatives are more suitable. However, in sections of formaldehyde-fixed

tissue reliable staining of BrdU can be obtained if the sections are pre-treated with pepsin (Schutte et al., 1987b). Second, in previous studies on the NE- and E-storing cells of the adrenal medulla, cryostat sections instead of sections from paraffin-embedded material were recommended. However, if formaldehyde fixation is performed by intracardial perfusion, most of the NE and E immunoreactivity is retained in paraffin-embedded tissue (Neelissen and Verhofstad, unpublished observation). These experiments also indicated that DBH and PNMT immunoreactivity is still present in paraffin sections of formaldehyde-fixed adrenal glands. As shown by Schutte et al. (1987a,b), cryostat as well as paraffin sections can be used for immunocytochemical detection of BrdU. Third, the proliferation rate of adrenal medullary cells has been shown to be rather low. Therefore, to obtain a sufficient number of BrdU-labeled cell nuclei within one section it was necessary to apply BrdU by continuous infusion using mini-osmotic pumps implanted into the peritoneal cavity. Finally, to give the BrdU antibody access to the BrdU incorporated into cell nuclei, DNA must be denatured to obtain single-stranded DNA. DNA denaturation procedures reported in the literature are based on the exposure of tissue sections to heat, alkaline, or acid (Soriano and Del Rio, 1991; Hayashi et al., 1988; Schutte et al., 1987b; Sugihara et al., 1986; Moran et al., 1985). More recently, a procedure using treatment of sections with the enzyme nuclease was reported. However, if cross-linking fixatives such as formaldehyde are used, additional deproteinizing by pepsin is needed (Dinjens et al., 1992; Montuenga et al., 1992). We have applied DNA denaturation with 2 N HCl because this procedure is most effective and because other procedures require an even stronger pepsin pre-treatment (Dinjens et al., 1992).

Using pepsin pre-treatment and HCl denaturation in paraffin sections from several organs, distinctly BrdU-stained cell nuclei could be observed. Our study confirms previous experiments by Dinjens

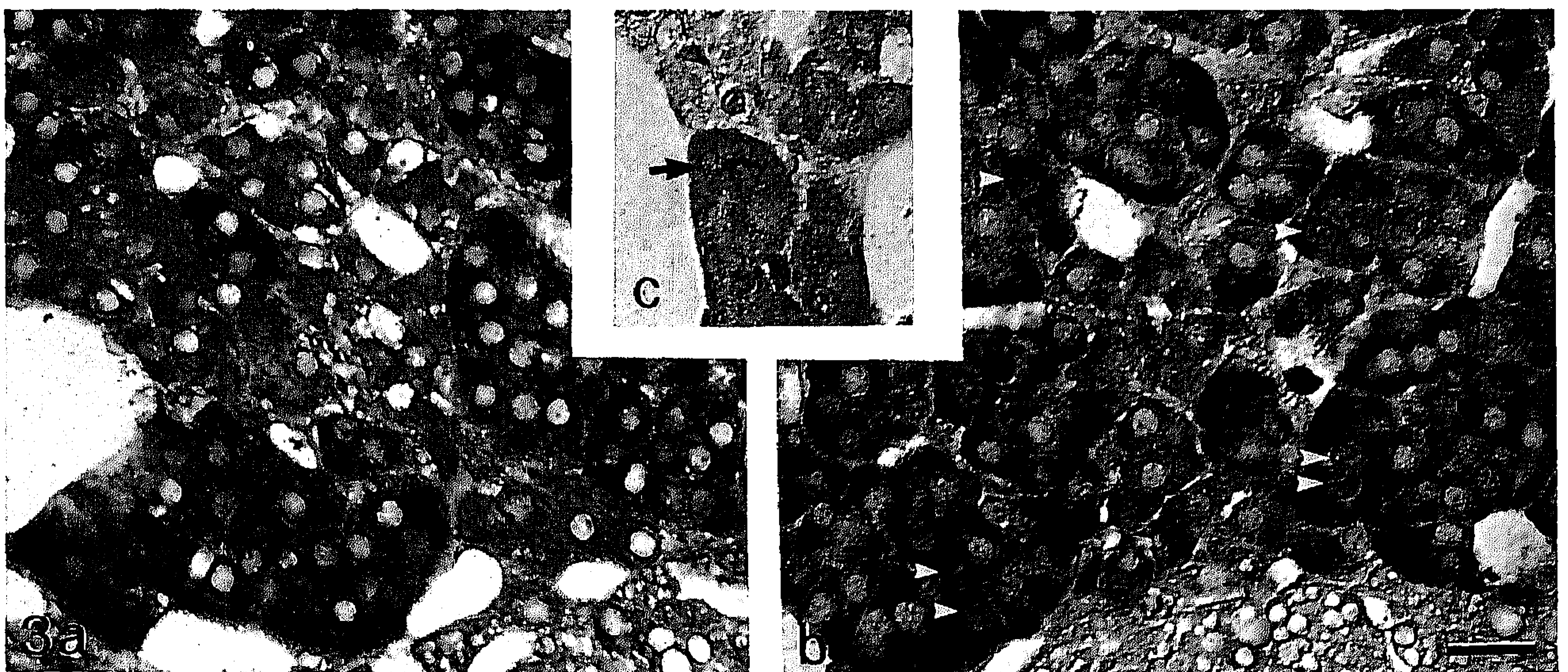


Figure 3. Rat adrenal glands exposed to BrdU for 2 weeks, fixed by perfusion with 4% paraformaldehyde, and embedded in paraffin. (a) Section stained for norepinephrine (red, Fast Red) and epinephrine (blue, Fast Blue). (b) Section stained for BrdU (brown, DAB), norepinephrine (red, AEC), and epinephrine (blue, Fast Blue). Only E cells show nuclei with BrdU incorporation (white arrowheads). In most cases these positive cell nuclei occur in pairs. (c) Section stained for BrdU (brown, DAB) and E (red, Fast Red). Mitotic figure in E-immunoreactive cell shows BrdU incorporation. Bar = 20  $\mu$ m.



et al. (1992) and Montuenga (1992) indicating that the optimal conditions with regard to pepsin and HCl pre-treatment might differ significantly in different tissues.

Based on a series of single experiments we developed a double immunoenzymatic staining procedure in which first NE, E, DBH, or PNMT is detected by an indirect alkaline phosphatase (AP) reaction with a Fast Red substrate followed by an indirect immunoperoxidase reaction developed with DAB to demonstrate BrdU. The AP-Fast Red reaction product appeared not to be affected by pepsin pre-treatment and HCl denaturation. Fast Blue or New Fuchsin as a substrate for AP and DAB or aminoethylcarbazole (AEC) as substrates for the peroxidase reaction also appeared to be resistant to pepsin pre-treatment and HCl denaturation. However, although other combinations are feasible, we prefer the use of Fast Red and DAB because of the high contrast of this combination. The sensitivity and localization obtained by the double-labeling procedure equal that of the single labeling of NE, E, DBH, PNMT, and BrdU. In addition, for triple labeling of NE, E, and BrdU and double labeling of NE and E, the sensitivity and localization of the immunoreactive material were the same as in the single-immunostaining experiments.

Although the present study was not aimed to examine the proliferation of the medullary cells in great detail, in the adrenal medulla of 9-week-old Wistar rats exposed for 2 weeks to BrdU some interesting observations were made. Sections stained according to the double- and triple-labeling procedures showed randomly distributed BrdU-positive nuclei throughout the medulla in both NE- and E-storing cells. Occasionally we identified mitotic figures, positively stained for BrdU, in cells that, based on the staining pattern of the cytoplasm, appeared to be fully differentiated, i.e., showed immunoreactivity to E, PNMT, or DBH. We did not find mitotic figures in cells stained for NE, most likely because mitotic figures are very rare. These findings indicate that in the adrenal medulla fully differentiated medullary cells are able to divide. However, it can not be excluded that part of the BrdU staining is the result of BrdU uptake by some kind of progenitor cell. Our observations confirm previous immunocytochemical studies using simultaneous staining of PNMT and BrdU (Tischler et al., 1989) and electron microscopic studies that revealed mitotic figures in cells containing both typical NE and E storage granules (Tischler et al., 1988). However, the advantage of our double- and triple-labeling procedures is that we can detect catecholamine-biosynthesizing enzymes as well as NE and E in cells that have incorporated BrdU. In particular, the applicability of antibodies to NE and E makes it possible to study cell proliferation in tissues of species containing biosynthesizing enzymes that do not crossreact with the currently available antibodies to DBH or PNMT (Verhofstad et al., 1980, 1983, 1985, 1989).

In conclusion, we have developed double- and triple-immunoenzymatic procedures that enable simultaneous staining of BrdU and NE- or E-storing cells. It is probable that the procedures described might also be applicable to other tissues containing catecholamines or other related monoamines.

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